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Virus Resistance in *Nicotiana benthamiana* Conferred by African Cassava Mosaic Virus Replication-Associated Protein (AC1) Transgene

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The replication-associated protein AC1 of the bipartite geminivirus African cassava mosaic virus (ACMV) is essential for viral DNA replication. Transient expression of AC1 or the truncated N-terminal portion of the protein caused a significant reduction in the level of viral DNA replication in *Nicotiana tabacum* protoplasts. *N. benthamiana* plants have been transformed with the *AC1* coding sequence cloned downstream of the enhanced cauliflower mosaic virus 35S promoter. Five lines produced detectable levels of the appropriate-sized *AC1*-specific transcript, although none was able to complement the systemic infection of an ACMV *AC1* mutant. However, all lines showed some level of resistance to ACMV infection; the majority of plants either remained asymptomatic or produced delayed and attenuated symptoms, and accumulated significantly reduced levels of viral DNA in comparison with infected control plants. In leaf disk assays, viral DNA replication was also reduced in transformed lines. None of the transformed lines showed resistance to the related geminiviruses tomato golden mosaic virus and beet curly top virus, demonstrating the specific nature of the interaction. Possible mechanisms for the resistance phenomenon are discussed.

Additional keywords: cassava mosaic disease, nonconventional resistance.

Geminiviruses are widely distributed plant DNA viruses that collectively cause economically important diseases in a wide range of cereal, vegetable, and fiber crops (reviewed by Efron et al. 1989; Brown and Bird 1992; Brown 1994). Cassava (*Manihot esculenta* Crantz) is cultivated widely throughout the tropics, primarily in Africa, and is the third largest carbohydrate source for human consumption (Fauquet and Fargette 1990). Cassava mosaic disease (CMD), considered to be the most devastating disease of cassava in Africa (Bock 1983; Fauquet and Fargette 1990), is caused by the geminivirus African cassava mosaic virus (ACMV; synonym, cassava latent virus) (Bock and Guthrie 1978; Bock and Woods 1983). ACMV is a typical whitefly-transmitted subgroup III virus (Mayo and Martelli 1993) with a bipartite genome (DNAs A and B) (Stanley 1983; Stanley and Gay 1983). Conventional breeding programs to control CMD have met with limited success because natural resistance, introduced by interspecific crosses with plants of limited agricultural value, is polygenic and recessive (Hahn et al. 1980), and all cultivars remain susceptible to virus infection.

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During geminivirus infection, the encapsidated single-stranded DNA (ssDNA) form is converted to double-stranded DNA (dsDNA) that in turn acts as a template for ssDNA synthesis by a rolling circle mechanism (Saunders et al. 1991; 1992; Stenger et al. 1991). Only one virus-encoded gene, *AC1* or *rep* (replication-associated protein), is required for viral DNA replication (Brough et al. 1988; Elmer et al. 1988; Etessami et al. 1991). During the course of replication, AC1 binds to a specific motif within the intergenic region (Fontes et al. 1992; 1994a, 1994b) and introduces a nick within the adjacent, highly conserved nonanucleotide TAATATTAC to initiate rolling circle replication (Laufs et al. 1995; Stanley 1995). Because the binding motif is located between the TATA box and transcription start site in the *AC1* promoter, AC1 binding causes transcriptional down-regulation of its own expression (Haley et al. 1992; Sunter et al. 1993; Eagle et al. 1994; Gröning et al. 1994). For ACMV, AC1 sequences responsible for repression have been mapped to the N-terminal 57 amino acids (Hong and Stanley 1995). Here, we have investigated the effect of transient expression of AC1 on viral DNA replication in *N. tabacum* protoplasts and the influence of an *AC1* transgene on systemic infection and symptom development in transgenic *N. benthamiana*.

RESULTS

ACMV DNA replication is inhibited by transiently expressed AC1.

The effect of AC1 on viral DNA replication was investigated by cotransformation of *N. tabacum* BY-2 protoplasts with DNA A (pCLV1.3A) and various cassettes expressing AC1 and mutant derivatives from the cauliflower mosaic virus (CaMV) 35S promoter (Fig. 1). DNA A alone replicated in this cell line to produce typical ssDNA and dsDNA forms (lane 1). A significant decrease in the level of viral DNA (70% average reduction [range 62 to 83%] as estimated by scanning densitometry of autoradiographs over three independent experiments), occurred in the presence of AC1 expressed from pAC1 (lane 2). Comparable reductions occurred in the presence of the AC1 mutants expressed from pMAC1-1 (69% average reduction [range 55 to 80%]) and pMAC1-2

(76% average reduction [range 75 to 76%]) (lanes 3 and 4, respectively). pMAC1-1 has the capacity to encode 57 N-terminal amino acids of AC1 fused to 93 amino acids from an adjacent reading frame, while a translational stop codon has been introduced in pMAC1-2 immediately after the 57 N-terminal amino acids of AC1. pMAC1-3, containing a translational stop codon immediately downstream of the *AC1* initiation codon, had little effect on viral DNA replication (8% average reduction [range 2 to 15%]) (lane 5), implying that AC1 expression rather than simply the presence of the *AC1* DNA per se was primarily responsible for reducing viral DNA levels.

Because the coding sequence for the putative gene *AC4* is located entirely within that of *AC1* (Etessami et al. 1991) and internal initiation within *AC1* has been demonstrated (Hong and Stanley 1995), the possibility remained that expression of AC4 from pAC1 could have an effect on viral DNA accumulation. However, viral DNA replication in *N. tabacum* protoplasts was largely unaffected in the presence of either intact AC4 (pAC4; lane 6) or an AC4 mutant truncated to 48 N-terminal amino acids (pMAC4-1; lane 7), demonstrating that this putative gene product does not contribute to the inhibitory phenomenon.

Transformation of *N. benthamiana*.

Seven *N. benthamiana* lines transformed with an *AC1* expression cassette cloned between the T-DNA borders of the binary vector pBin19 (pBin35S-AC1-polyA) have been produced. The phenotype of each transformant was normal, and the presence of an *AC1*-specific transgene in each line was verified by PCR amplification of a 1.1-kbp viral DNA fragment from genomic DNA extracted from F_1 progeny (Fig. 2A). The analysis of kanamycin sensitivity in the F_1 and F_2 progeny suggested that five lines (005, 019, 020, 021, and 036) contained a single copy of the transgene and two lines (001 and 002) contained two copies. Copy numbers were confirmed by *Eco*RV and *Hin*dIII restriction analysis and Southern blotting of genomic DNA (data not shown). An additional line (038) was obtained that was transformed with a single copy of the control cassette lacking the *AC1* coding sequence (pBin35S-polyA). To investigate transgene expression, transcripts isolated from the transgenic lines were analyzed by blot hybridization using an *AC1*-specific probe (Fig. 2B). A single *AC1*-specific transcript of approximately 1.3 kb was detected in extracts of lines 001, 005, 019, 021, and 036, but not in lines 002, 020, 038 (control) and nontransformed *N. benthamiana*. PCR amplification and sequence analysis of the transgene in line 036 confirmed that the *AC1* coding sequence remained intact. Homozygous F_2 lines 005, 019, 020, 021, 036, and 038, and kanamycin-resistant F_2 lines 001 and 002, were used for further analysis.

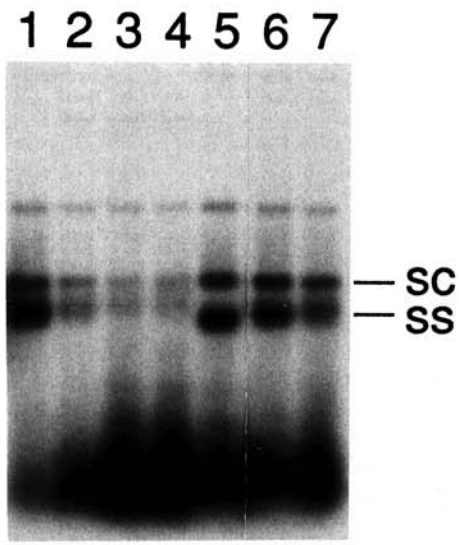

Fig. 1. The effect of expression of AC1 and its mutant derivatives on ACMV DNA replication in *Nicotiana tabacum* BY-2 cells. Total nucleic acids were extracted from cells transfected with ACMV DNA A (pCLV1.3A) either alone (lane 1) or in the presence of pAC1 (lane 2), pMAC1-1 (lane 3), pMAC1-2 (lane 4), pMAC1-3 (lane 5), pAC4 (lane 6) and pMAC4-1 (lane 7). Equal amounts of total nucleic acids (5 µg) were loaded in each lane and the blot was hybridised to a DNA A-specific probe. The positions of single-stranded (ss) and supercoiled (sc) viral DNAs are indicated.

The *AC1* transgene is unable to trans-replicate viral DNA in planta.

It has been demonstrated that ACMV *AC1* mutants are unable to systemically infect *N. benthamiana* and cannot replicate in *N. tabacum* protoplasts, although *AC1* function can be complemented in *trans* by a coinfecting virus (Etessami et al. 1991). However, none of the transgenic lines supported a systemic infection when plants were mechanically coinoculated with an *AC1* mutant and DNA B (supplied as pCLVAC1-2 insert and pCLV2B), demonstrating that the transgene is unable to complement the mutation in planta. Furthermore, although DNA A (from pBin1.3A) replicated in leaf disks derived from each of the transgenic lines (for example, see Fig. 3), replication of the *AC1* mutant (from pBin1.3AC1-2) was not detected and the transgene was unable to trans-replicate DNA B (from pBin2B) (data not shown).

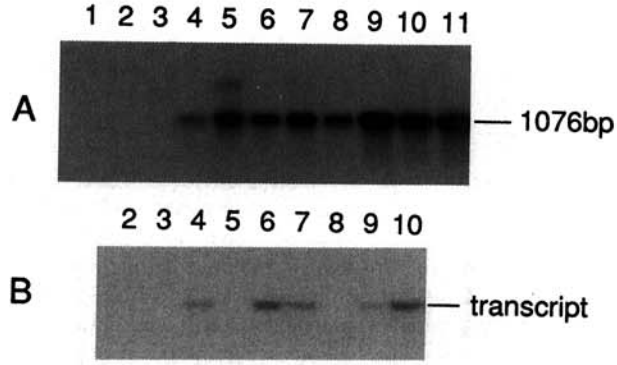

Fig. 2. Transgene expression in *AC1*-transformed *Nicotiana benthamiana*. **A,** A fragment of the transgene was amplified from total nucleic acid extracts by PCR using *AC1*-specific primers, and detected by Southern blot analysis using an *AC1*-specific probe. Samples were from a nontransformed plant (lane 2) and lines 038 (lane 3), 001 (lane 4), 002 (lane 5), 005 (lane 6), 019 (lane 7), 020 (lane 8), 021 (lane 9), and 036 (lane 10). In lane 1, no template DNA had been added prior to PCR amplification, and in lane 11, the authentic 1,076-bp fragment has been amplified from an extract of a nontransformed plant infected with ACMV. **B,** Total RNA (20 µg) extracted from young leaves was analyzed by blot hybridization using an *AC1*-specific probe. The transcript size (approximately 1.3 kb) was estimated by comparison with an RNA ladder (Gibco BRL). Samples were from the plant lines described above.

The *AC1* transgene has an adverse effect on ACMV systemic infection.

Transgenic plants were mechanically inoculated with ACMV cloned components, supplied as pJS092 insert and pCLV2B (Table 1). Plants were regularly screened for symptom development, and symptom severity was graded from 1 (mild) to 4 (wild-type) as previously described (Stanley et al. 1990). All inoculated plants in the control line 038, lacking *AC1* sequences, produced symptoms of wild-type severity and timing that were indistinguishable from those in nontransformed plants. In contrast, most other transgenic lines were less susceptible to ACMV infection as judged by the number of plants showing mild and delayed symptoms and, in the case of lines 001, 005, 019, and 036, by the high proportion of asymptomatic plants. Of these, line 036 was least susceptible to infection, with 68% of the plants remaining asymptomatic. The notable exceptions to this were lines 002 and 020 that produced predominantly wild-type symptoms. The majority of transgenic plants became infected when inoculated with either tomato golden mosaic virus (TGMV) or beet curly top virus (BCTV) (Table 1), and all infected plants produced wild-type symptoms that were identical to those produced in nontransformed plants.

The level of viral DNA in ACMV-infected plants of line 036 was investigated by dot blot analysis of young leaves sampled 3 weeks after the virus challenge, using component-specific probes (Fig. 4). Viral DNA was either not detected or, occasionally, detected at low levels (less than 10% of wild-type) in asymptomatic plants. Viral DNA levels were variable in leaves infected at different stages of development, although the accumulation of both genomic components generally increased with symptom severity, consistent with an earlier report on this particular virus/host combination (Stanley et al. 1990). Hence, the presence of the *AC1* transgene caused a general reduction in viral DNA accumulation in systemically infected tissues, resulting in symptom attenuation.

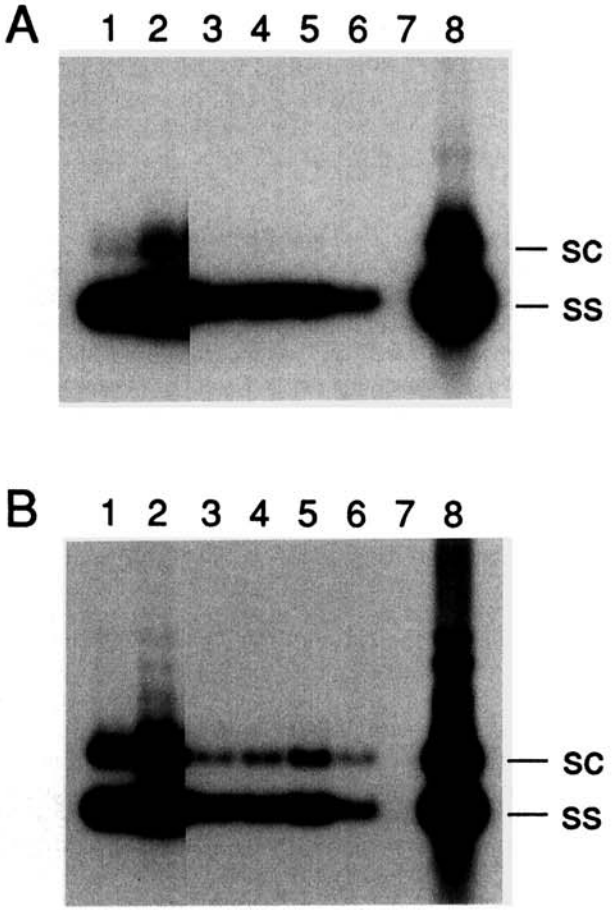

Fig. 3. ACMV DNA replication in *AC1*-transformed *Nicotiana benthamiana* leaf disks. Total nucleic acids were extracted from leaf disks from a nontransformed plant (lane 1) and lines 038 (lane 2) and 036 (lanes 3-6) coagroinoculated with pBin1.3A and pBin2B, from noninoculated 036 leaf disks (lane 7) and from a systemically infected plant (lane 8). The inoculated leaf disks from line 036 shown in lanes 3-6 originated from four different F_2 plants. Equal amounts of total nucleic acids (5 µg) were loaded in each lane and the blots were hybridized to probes specific to DNA A (A) and DNA B (B). Both A and B are composites from single autoradiographs. The positions of single-stranded (ss) and supercoiled (sc) viral DNAs are indicated.

Table 1. Infectivity of geminiviruses and symptom production in control and transgenic *Nicotiana benthamiana*

Plant	ACMV[a]						
line	0	1	2	3	4	TGMV[b]	BCTV[b]
001	11	2	6	4	3	8/8	8/8
002	1	3	0	1	21	10/10	9/10
005	10	1	6	6	3	10/10	10/10
019	13	4	10	7	1	8/8	8/8
020	0	0	0	0	21	10/10	10/10
021	5	1	5	11	4	9/9	9/9
036	25	4	5	1	2	10/10	10/10
038	0	0	0	0	21	10/10	10/10
NT[c]	0	1	0	0	20	10/10	9/10

[a] The number of plants showing a particular symptom type associated with ACMV infection, ranging from mild (grade 1) to wild-type severity (grade 4) as defined by Stanley et al. (1990), are given. Asymptomatic plants are grade 0.

[b] All plants infected with TGMV and BCTV showed symptoms of wild-type severity.

[c] Nontransformed.

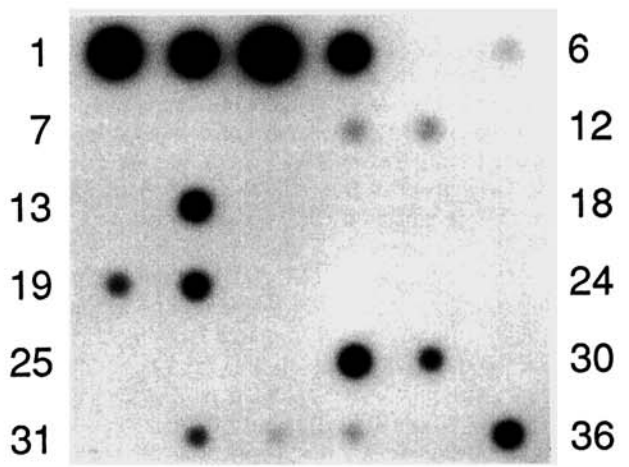

Fig. 4. Dot blot analysis of ACMV DNA levels in *AC1*-transformed *Nicotiana benthamiana*. Upper leaves were sampled from nontransformed plants (1 and 2) and plants of lines 038 (3 and 4) and 036 (5-36), and probed for viral DNA. Plants were either asymptomatic (5-9, 12, 13, 15-18, 21-27, 30, 31, and 35) or developed systemic symptoms of grade 1 (mild; 10, 11, and 34), grade 2 (19, 20, 32, 33, and 36), grade 3 (14 and 29) or grade 4 (wild-type; 1-4 and 28) severity, as defined by Stanley et al. (1990). Similar results were obtained using probes specific to either DNA A or DNA B.

To further investigate the effect of the *AC1* transgene on viral DNA levels, leaf disks derived from transgenic lines 036 and 038, as well as from nontransformed plants, were coagroinoculated with cloned ACMV components (pBin1.3A and pBin2B), and the resulting viral DNA forms were analyzed by Southern blotting (Fig. 3). Levels of ssDNA and dsDNA forms of both genomic components varied slightly between batches of leaf disks from different plants, presumably due to sampling leaves at different stages of development. However, the level of viral DNA that accumulated in line 036 (lanes 3 to 6) is clearly significantly reduced (90% average reduction [range 89 to 95%]) compared with that in leaf disks from line 038 (lane 2), which lack the *AC1* sequence.

DISCUSSION

Our results suggest that transient expression of intact AC1 or an N-terminal fragment of the protein significantly inhibits ACMV DNA accumulation in *N. tabacum* protoplasts. The *AC4* coding sequence, conserved in subgroup III geminiviruses and located entirely within *AC1*, does not contribute to the inhibitory effect. It is noteworthy that no role has been assigned to ACMV *AC4*, and it is dispensible for infection of *N. benthamiana* (Etessami et al. 1991). Furthermore, transiently expressed AC4 does not affect *AC1* expression in *N. tabacum* protoplasts (Hong and Stanley 1995).

On the strength of these preliminary results, we were interested to know if constitutively expressed AC1 might have an adverse effect on ACMV infection in transgenic plants. Accordingly, *N. benthamiana* was transformed with the *AC1* coding sequence under the control of the CaMV 35S promoter, and five independent lines were obtained that expressed detectable levels of the transgene transcript. All of these lines were less susceptible to ACMV infection as judged by the reduction in the number of plants becoming infected and the decrease in both viral DNA accumulation and severity of symptoms when compared with controls. Two lines transformed with *AC1* sequences but unable to express detectable levels of transcript, and one line transformed with just the 35S promoter and polyadenylation sequences were highly susceptible to ACMV infection, implying that *AC1* transgene expression is responsible for conferring resistance to infection.

Geminivirus *rep* genes are multifunctional, participating in both viral DNA replication and the control of gene expression. TGMV AL1 binds with high specificity to motifs located between the consensus TATA box and the initiation site for complementary-sense transcription (Fontes et al. 1992; 1994a; 1994b) prior to introducing a nick in the virion-sense strand and binding covalently to the exposed 5′ terminus during the initiation of DNA replication (Laufs et al. 1995; Stanley 1995). Presumably, binding at this position is responsible for the observed down-regulation of complementary-sense gene expression observed for both TGMV (Sunter et al. 1993; Eagle et al. 1994; Gröning et al. 1994) and ACMV (Haley et al. 1992; Hong and Stanley, 1995). On the basis of our current understanding of *rep* functions, several possible explanations may be proposed for the effect of AC1 on viral DNA accumulation in *N. tabacum* protoplasts. Constitutive expression of AC1 could result in its unregulated binding to the origin of replication, disrupting processive viral DNA synthesis during rolling circle replication and/or gene expression by competing for cellular transcription factor binding sites. Alternatively, it could adversely affect the integrity of the viral DNA by introducing nicks at cryptic motifs, in a manner similar to that described for wheat dwarf virus (Heyraud et al. 1993). If AC1 functions as a multimer, as has been shown for the functionally equivalent initiator protein of plasmid pT181 (Thomas et al. 1990; Rasooly et al. 1994), it could affect viral DNA replication by disturbing the equilibrium between monomeric and multimeric forms.

We do not yet know if *AC1*-mediated pathogen-derived resistance in transgenic plants is the direct effect of expression of AC1 protein, if it occurs at the level of transcription in a mechanism resembling cosuppression (reviewed by Lomonossoff 1995), or a combination of both. Both alternatives can accommodate the virus-specific nature of the phenomenon. It was somewhat surprising to find that none of the lines were able to functionally complement an *AC1* mutant, particularly as transgenic *N. benthamiana* plants expressing high levels of TGMV AL1 (the homolog of ACMV AC1) have been shown to produce symptoms of wild-type severity when challenged with an *AL1* mutant (Hanley-Bowdoin et al. 1990). This suggests either that the timing, location, or level of AC1 expression is unsuitable for mutant complementation or that selection against a functional transgene has occurred during plant transformation and regeneration. Given the proposed function of geminivirus *reps* in controlling the cell environment (Nagar et al. 1995; Xie et al. 1995), AC1 may well exert an adverse effect on the development of transgenic plants. Consistent with this view, of the seven plant lines that contain the *AC1* transgene, two (002 and 020) are unable to synthesize detectable levels of transcript, demonstrating that the transgene has undergone some degree of modification. However, we confirmed that the *AC1* coding sequence in line 036 remained intact, implying that the transgene has the capacity to express functional protein from the *AC1*-specific transcript present in this particular line.

Several approaches to control geminivirus diseases using contemporary genetic engineering have recently been developed. Transgenic *N. benthamiana* containing a naturally occurring ACMV defective interfering (DI) DNA is less susceptible to ACMV infection due to a reduction in the accumulation of viral DNA (Stanley et al. 1990; Frischmuth and Stanley 1991). Resistance is confined to closely related strains of ACMV owing to the need for *AC1*-mediated trans-replication of the DI DNA. Similarly, a DI DNA has been shown to confer a degree of resistance to BCTV infection in transgenic *N. benthamiana* and the effect is again virus-specific (Frischmuth and Stanley 1994; Stenger 1994). Transgenic *N. tabacum* expressing antisense RNA targeted against TGMV *AL1* is less susceptible to TGMV infection (Day et al. 1991), and resistance specificity depends on the level of homology between the antisense RNA and its target as well as on the spacing of homologous sequences (Bejarano and Lichtenstein 1994). Finally, transgenic tomato transformed with two overlapping virion-sense genes (coat protein gene *V1* and *V2*) of tomato yellow leaf curl virus is reported to be resistant to virus infection (Kunik et al. 1994). The use of an *AC1* transgene described here represents a novel strategy for the production of geminivirus-resistant plants. Having demonstrated that an N-terminal fragment of AC1 also adversely affects viral DNA

replication in *N. tabacum* protoplasts, it may prove equally effective as intact AC1 in conferring resistance to ACMV in transgenic plants.

MATERIALS AND METHODS

Clone construction.

The construction of pAC1 and pAC4, containing the *AC1* and *AC4* coding sequences, respectively, under the control of the enhanced CaMV 35S promoter and polyadenylation sequences in the plant expression vector pJIT163 (Guerineau et al. 1992), and their mutant derivatives pMAC1-1, pMAC1-2, pMAC1-3, and pMAC4-1, has been described (Hong and Stanley 1995). The viral DNA inserts of pAC1 and pJIT163 were cloned into pBin19 (Bevan 1984) using flanking *Sst*I and *Xho*I sites, to produce pBin35S-AC1-polyA and pBin35S-polyA, respectively. The construction of infectious clones of ACMV genomic components (full-length DNA A clone pJS092, partial repeats of DNA A [pCLV1.3A and pBin1.3A] and dimers of DNA B [pCLV2B and pBin2B]), an ACMV *AC1* mutant (pCLVAC1-2), and infectious clones of TGMV (csTA1.6 and csTB1.4) and BCTV (pBin1.2) has been described (Stanley 1983; Briddon et al. 1989; Klinkenberg et al. 1989; Etessami et al. 1991; von Arnim and Stanley 1992). The *AC1* mutant was cloned from pCLVAC1-2 as a partial repeat in pBin19 essentially as described for pBin1.3A, to produce pBin1.3AC1-2 (generously provided by K. Saunders). Viruses were held and manipulated under MAFF licence numbers PHF 1185A/68(21) and PHF 1185B/17(111) under the Plant Pests (Great Britain) Order 1980.

Plant transformation and analysis.

Clones pBin35S-AC1-polyA and pBin35S-polyA were mobilized into *Agrobacterium tumefaciens* LBA4404 (Hoekema et al. 1983) by triparental mating (Ditta et al. 1980). *N. benthamiana* was transformed using a leaf disk method (Horsch et al. 1985; Horsch and Klee 1986) and transformants were selected for their resistance to 100 μg of kanamycin per ml and 500 μg of carbenicillin per ml. Following self-fertilization, F_1 and F_2 progenies were tested for antibiotic sensitivity by germinating seeds on 500 μg of kanamycin per ml. The presence of the *AC1* transgene was verified by PCR amplification from total DNA extracted from F_1 progeny using *AC1*-specific primers corresponding to nucleotides 1680 to 1697 (virion-sense) and 2756 to 2737 (complementary-sense) of DNA A. PCR products were fractionated by agarose gel electrophoresis, transferred to Hybond N$^+$ (Amersham International) with a pressure blotter (Stratagene) according to the manufacturers' recommendations, and hybridized to an *AC1*-specific probe corresponding to DNA A nucleotides 1658 to 2766, prepared by random priming (Feinberg and Vogelstein 1983). The integrity of the transgene in line 036 was investigated by sequence analysis of the PCR product using a T7 sequencing kit (Pharmacia) and primers specific to the viral DNA. The copy number of integrated DNA was estimated from kanamycin sensitivity data and by blot hybridization analysis of plant genomic DNA (Dellaporta et al. 1983). To analyze transgene expression, total cellular nucleic acids were extracted from leaves and high molecular weight RNAs were isolated by salt precipitation as described by Covey and Hull (1981). RNA aliquots (20 μg) were fractionated by agarose gel electrophoresis in 2.2 M formaldehyde (Sambrook et al. 1989), blotted, and probed with a radiolabeled *AC1*-specific fragment.

Maintenance and inoculation of plants.

Plants were maintained in accordance with the requirements of the Advisory Committee on Genetic Manipulation, in an insect-free glasshouse at 25°C (reduced to 20°C at night) with supplementary lighting to give a 16-h photoperiod. Plants were either mechanically inoculated on Celite-dusted expanding leaves (Stanley 1983) using DNA A inserts of the wild-type clone (pJS092) or *AC1* mutant (pCLVAC1-2), together with undigested DNA B clone pCLV2B, or agroinoculated with TGMV and BCTV (Briddon et al. 1989; von Arnim and Stanley 1992). Plants were screened daily for the appearance and severity of symptoms.

Detection of viral nucleic acids in plants.

Total nucleic acids were extracted from upper leaves 3 weeks postinoculation as described by Covey and Hull (1981), and 2.5-μg aliquots were denatured with 50 mM NaOH, neutralized with 1/10 volume of 3 M sodium acetate (pH 5.2) and spotted onto Hybond N$^+$ (Amersham International). Viral DNAs were detected by hybridization with radiolabeled probes specific to DNA A or DNA B (Klinkenberg et al. 1989).

Analysis of viral DNA replication in *N. tabacum* protoplasts.

Protoplasts were prepared from suspension cultures of *N. tabacum* cell line BY-2 (Nagata et al. 1992) and transformed as described by Hong and Stanley (1995). Transformed protoplasts were cultured in 5 ml of PCM at 25°C in the dark for 72 h prior to harvesting and nucleic acid extraction. Protoplasts were harvested, washed once with 0.4 M mannitol, and resuspended in 5 ml of ice-cold extraction buffer (50 mM Tris-HCl, 25 mM EDTA, pH 8.0) containing 1% SDS. After 5 min of incubation at 65°C, the mixture was extracted once with phenol/chloroform (1:1) and once with chloroform. Total nucleic acids were precipitated with ethanol, washed with 70% ethanol, dried under vacuum, and dissolved in 50 μl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA samples were fractionated by agarose gel electrophoresis in 40 mM Tris-acetate, 20 mM sodium acetate, 2 mM EDTA, pH 7.5 (TNE buffer). After depurination, denaturation, and neutralization, nucleic acids were transferred to Hybond N$^+$ (Amersham International) and hybridized to a radiolabeled DNA A-specific probe. Viral DNA levels were estimated by absorbance scanning of autoradiographs using a Chromoscan 3 (Joyce Loebl).

Analysis of viral DNA replication in transgenic *N. benthamiana* leaf disks.

The ability of ACMV to replicate in leaf disks derived from transgenic *N. benthamiana* was assayed as described by Klinkenberg et al. (1989) in accordance with established protocols (Horsch et al. 1985; Horsch and Klee 1986). Total nucleic acids were extracted from batches of five to seven leaf disks harvested 9 days after agroinoculation, and 5-μg aliquots were analyzed by agarose gel electrophoresis in TNE

buffer and blot hybridization using radiolabeled probes specific to DNA A or DNA B.

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